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Exploration of the Genetic Diversity and Differentiation of *Craspedacusta sowerbyi* in Guizhou Province

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Key words
***Craspedacusta sowerbyi*, Genetic diversity, Genetic structure, Guizhou Province, Genetic differentiation**

ABSTRACT

To understand the genetic diversity and genetic differentiation status of *Craspedacusta sowerbyi* Lankester, 1880 in Guizhou Province, genetic diversity and genetic structure analyses of three geographical populations of *C. sowerbyi* in Guizhou Province were performed using the results of PCR amplification and direct sequencing based on the mitochondrial DNA *COI* gene. Sixty-two individuals displayed four haplotypes, and the overall haplotype diversity (Hd) and nucleotide diversity (Pi) of the three *C. sowerbyi* groups were 0.531 and 0.00067, respectively. Analysis of molecular variance (AMOVA) showed that inter- and intrapopulation variation accounted for 41.63039% and 58.36961%, respectively, of the total variation, and the genetic variation coefficient (F_{st}) was 0.05268. The gene flow (Nm) values between the three *C. sowerbyi* populations were <1. The results of neutrality, Fu's Fs and Tajima's D tests did not show significant negative values. The overall nucleotide diversity of the three *C. sowerbyi* populations in Guizhou Province was low, especially in the Xiuwen County population, the genetic variation mainly occurred within the populations.

INTRODUCTION

Craspedacusta, the only small jellyfishes that can survive in fresh water (He and Xu, 2003), belong to family Olindiidae of class Hydrozoa. *Craspedacusta* have been on Earth for 550 million years or more (He and Xu, 2003) and are veritable living fossils. In the life history of *Craspedacusta,* there are two states, the jellyfish type and hydra type, and alternating generations of asexual reproduction and sexual reproduction. There are currently three species of *Craspedacusta* (Hu, 2005; Su and Wang, 2009) in the world: *Craspedacusta sowerbyi*, *Craspedacusta iseanum* and *Craspedacusta sinensis*. Peach blossom jellyfish are found in Israel, Ireland, Chile, the United States (Caputo *et al*., 2013; Fedorkov, 2002; Gasith *et al*., 2011; Minchin *et al*., 2016) and many other countries. China is the country with the widest distribution of peach blossom jellyfish, with these jellyfish found in 19 regions (Wan *et al*., 2013). Because peach blossom jellyfish spend a very short amount of time at the water surface and are rarely found in the same water body for several consecutive years, the discovery of peach blossom jellyfish in the wild was a great accident. Fortunately, from 2020 to 2021, we collected wild peach blossom jellyfish (*Craspedacusta sowerbyi*) in three different places in Guizhou, giving us the opportunity to conduct population genetics research on this ancient species. Further research is needed on the world's only freshwater jellyfishes to increase awareness of these species.

Guizhou province is located in southwestern China on the Yunnan-Guizhou Plateau, which is an underdeveloped area of China. The agriculture is still based on traditional farming, industrial development started late, and the number and scale of industries are not large (Yi and Zhang, 2017). For these reasons, the ecological environment in Guizhou province is

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in a relatively good state, and the frequency of peach blossom jellyfish is relatively high. From 2011 to 2021, our laboratory was fortunate to have found peach blossom jellyfish at different times and places in Guizhou province (a total of six times) and carried out related research. We explored the morphological characteristics and movement mode of peach blossom jellyfish (Liang *et al*., 2011), its identification in Fuquan city (Tao *et al*., 2019), and its gonadal development (Tao *et al*., 2019). From 2020 to 2021, groups of peach blossom jellyfish were found in Zhazuo Town (XW) of Xiuwen county, Guizhou province, and Wanshan Town (WS) and Huangdao Township (HD) in Wanshan district and were molecularly identified as *Craspedacusta sowerbyi*.

Mitochondrial DNA (mtDNA) is characterised by maternal inheritance and a fast evolutionary rate. The cytochrome C oxidase I subunit (*COI*) gene is an important member of the mitochondrial oxidative respiratory chain, with a simple structure, fast evolution, almost no recombination, a large amount of evolutionary information and other characteristics (Amor *et al*., 2014), and it is an important gene that reflects the genetic diversity and phylogenetic evolution of aquatic animals. Dong *et al*. (2016) selected *COI* gene fragments to study the genetic structure of *Nemopilema nomurai* and found that no significant genetic differentiation occurred between the five populations. Govindarajan *et al*. (2006) found that the *COI* base replacement rate of *Obelia geniculata* was high and proposed that the *COI* gene was suitable for taxonomic studies of *Hydra* species. In addition, the *COI* gene is easy to amplify and sequence (Liu and Dong, 2018), making it suitable for molecular genetic research on jellyfish and stingrays. Miu *et al*. (2017) selected mitochondrial *COI* genes to study the genetic diversity of toxic jellyfish off the coast of Thailand.

Monitoring the genetic diversity and genetic structure of small biological populations is important for preserving their genetic diversity and gene pool uniqueness (Fornal *et al*., 2021). In November 2020, June 2021 and September 2021, peach blossom jellyfish were fortunately collected in Zhazuo Town (XW) in Xiuwen County, Wanshan Town (WS) in Wanshan District and Huangdao Township (HD) in Wanshan District. Based on the mitochondrial *COI* gene, we analysed the genetic diversity and genetic structure of these three populations of peach blossom jellyfish to shed light on the current status of the germplasm resources and genetic structure of this jellyfish, enrich the data available for this jellyfish, and provide basic data for understanding and protecting this ancient freshwater jellyfish.

MATERIALS AND METHODS

Materials

Due to the very short time that jellyfish appeared on the water surface, the jellyfish at the three sites in this study were all found by chance. The environment of the three sampling points is shown in Supplementary Figure 1. The rest of the sampling information is shown in Table I. To avoid residual plankton in the digestive cavity of the jellyfish from interfering with the purity of the extracted genomic DNA, the digestive cavity was separated on site with tweezers, and the umbrella portion of the body was stored in absolute ethanol at -20 °C for cryopreservation and brought back to the laboratory. The peach jellyfish in these three places were all identified as Soxhlet peach jellyfish (*Craspedacusta sowerbyi*).

PCR amplification of COI *gene*

The total DNA of the peach blossom jellyfish was extracted by the animal genome extraction kit of Qingke Biotechnology Co., Ltd. The *COI* sequence was amplified using universal primers, and the amplified fragment length was approximately 750 bp. The primers *COI*1 5'-TCAACCACCCACAAAGACATTGGCAC-3' *COI*2 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' were synthesized by Qingke Biotechnology. The total volume of the PCR system was 25 µL, including 1 µL of template DNA, 1 µL of upstream and downstream primers, and 22 µL of Gold Mix (manufactured by Qingke Biotechnology Co., Ltd.). PCR amplification conditions included predenaturation at 98 °C for 3 min; denaturation at 98 °C for 10 s, annealing at 58 °C for 10 s, and extension at 72 °C for 30 s, 35 cycles; and a final extension at 72 °C for 1 min. The product was detected by gel electrophoresis with 1% agarose, and the length and concentration of the product were observed with a gel imaging system.

Table I. Sampling information.

Group	Time	Sample capacity (N)	Sampled rice field (Chunk)	Latitude and longitude
XW	2020-11	12	The town of Zhazuo in Xiuwen county	E106°45.96′57.49″, N29°50.40′24.28″
WS	2021-06	25	Wanshan Town in Wanshan district	E109°13.55′32.82″, N27°31.33′19.55″
HD	2021-09	25	Zodiac Township in Wanshan district	E109°17′1.49″, N27°27.84′50.48″.

Data statistics and analysis

The sequencing results were aligned and manually adjusted using Clustal X 1.81 software (Thompson *et al*., 1997). MEGA v6.0 software was used to calculate the base content, identify variant sites, and determine genetic distances between various groups, and the neighbour-joining method was used to construct an evolutionary tree (Tamura *et al*., 2013). DnaSP v5.0 software was used to calculate haplotype diversity (Hd) and nucleotide diversity (Pi) (Lechner *et al*., 2011). The genetic differentiation index (F_{st}) was calculated and analysis of molecular variation (AMOVA) was performed using Arlequin v3.5 to test for significance using 1000 permutations, and the neutrality test was performed to obtain Fu's Fs and Tajima's D values (Excoffier and Lischer, 2010). Gene flow was calculated using the formula $Nm \approx (1-F_{st})/4Fst$. The haplotype network diagram was constructed using Network 5.0 (Schmidt-Roach *et al*., 2013).

RESULTS

Base composition of COI genes in three populations of jellyfish

A total of 62 Soxhlet jellyfish mitochondrial DNA *COI* genes were amplified and sequenced from the 3 geographic populations XW, WS and HD. The unstable sequences were trimmed at both ends, and the resulting 849 bp sequences were reserved for further analysis. The average contents of the 4 bases in the *COI* sequences of the 3 Soxhlet jellyfish populations are shown in Table II, including A (29.6%), T (21.3%), C (21.7%), and G (27.4%); the (A+T) content was 50.9%, and the (C+G) content was 49.1% (Table I).

Genetic diversity parameters based on COI sequences

The genetic diversity parameters based on the *COI* sequence are shown in Table III. The three Soxhlet jellyfish populations had a total of 5 defined haplotypes, and the XW population had the lowest haplotype diversity (Hd) and nucleotide diversity (Pi), measuring 0.30324 and 0.00036, respectively. The haplotype diversity (Hd) and nucleotide diversity (Pi) of the Huangdao Township population were the highest, at 0.59795 and 0.00080, respectively. The results of Fu's Fs and Tajima's D tests showed no significant negative values in any of the three populations (P<0.05), this shows that the three populations have not expanded in history.

Table II. *COI* sequence base composition of 3 *C. sowerbyi* populations (n=62).

Group	Gene	Base composition/%					
		A	T	C	G	(A+T)	(G+C)
XW	*COI*	29.7	21.3	21.7	27.4	51.0	49.1
WS	*COI*	29.6	21.3	21.7	27.4	50.9	49.1
HD	*COI*	29.6	21.3	21.7	27.4	50.9	49.1
Mean	*COI*	29.6	21.3	21.7	27.4	50.9	49.1

For details of groups, see Table I.

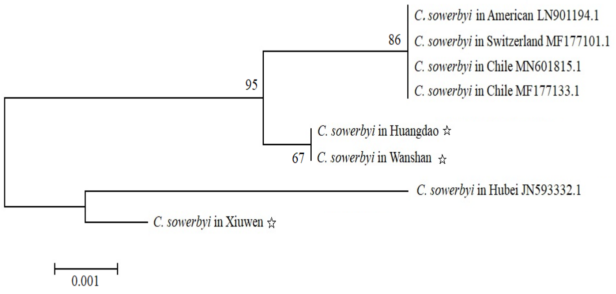

Fig. 1. *COI* gene-based NJ tree of *C. sowerbyi.*

Peach jellyfish NJ phylogenetic tree based on the COI *gene*

The *COI* gene-based NJ phylogenetic tree is shown in Figure 1, where the eight geographic populations of freshwater jellyfish are collectively divided between two large branches. The *C. sowerbyi* of Xiuwen and Hubei populations gathered onto a large branch. The *C. sowerbyi* population in Huangdao Township and that in Wanshan Town gathered onto a branch, and they also formed a branch with the *C. sowerbyi* populations from the United States, Switzerland and Chile.

Table III. Genetic diversity parameters of mitochondrial *COI* sequences in the *C. sowerbyi* populations (n=62).

Group	Gene	Haploid number	Haplotype diversity Hd	Nucleotide diversity Pi	Fu's Fs	Tajima's D
XW	*COI*	2 (Hap1-2)	0.30324	0.00036	0.29736	-0.19492
WS	*COI*	2 (Hap1-2)	0.52061	0.00061	1.60576	1.59799
HD	*COI*	2 (Hap1-4)	0.59795	0.00080	-0.66906	-0.35791
Mean	*COI*	2 (Hap1-4)	0.53137	0.00067	-	-

Note: * 0.01<P<0.05;** P<0.01. For details of groups, see Table I.

Genetic differentiation between three geographical populations of peach blossom jellyfish in Guizhou province

Based on the *COI* sequences, the genetic distances between and within the populations from Zazuo town, Xiuwen County, and Wanshan town and Zodiac township in Wanshan District are shown in Table IV, and the average genetic distance between the three groups was only 0.0010. The genetic distances within the three populations were 0.0000, 0.0010, and 0.0010, respectively (Table IV).

Table IV. Genetic distances between and within three *C. sowerbyi* populations based on *COI* sequences.

Population	Gene	XW	HD	WS
XW	*COI*			
HD	*COI*	0.001		
WS	*COI*	0.001	0.001	
Genetic distance within the group	*COI*	0.000	0.001	0.001

For details of population see Table I.

Table V. F_{st} values and gene flow (Nm) between 3 *C. sowerbyi* populations.

Population	Gene	Xiuwen	Huangdao	Wanan
Xiuwen	*COI*		1.63537	1.01040
Huangdao	*COI*	0.13260		-7.81201
Wanan	*COI*	0.19835	-0.03306	

Based on the *COI* sequences, the genetic differentiation between the XW, WS and HD populations is shown in Table V. The genetic differentiation coefficients (F_{st}) between the three populations were between -0.03306 and 0.19835, and the F_{st} value between the XW population and the WS population was the largest, at 0.19835.

Based on the *COI* sequences, the gene exchange between the XW, WS and HD populations is shown in Table V. The gene flow (Nm) between the three populations was between -7.81201 and 1.63537, and that between the XW population and the WS population was the largest, at 1.63537.

Based on the *COI* sequences, the AMOVA results are shown in Table V, and the degrees of freedom between and within the groups are 2 and 63, respectively. The molecular variability rates between and within the populations were 5.27% and 94.73%, respectively, and the molecular variation mainly occurred within populations. The overall genetic differentiation coefficient (F_{st}) of the populations was 0.05268 (Table VI).

DISCUSSION

Genetic diversity of the peach blossom jellyfish population in Guizhou province

The three groups (XW, WS and HD) each showed only 2 haplotypes. Referring to Was et al. (1998) haplotype diversity and nucleotide diversity values of 0.5 and 0.005 are the high and low standards, respectively (Grant and Bowen, 1998). The nucleotide diversity (Pi) of each population was low. This low nucleotide diversity was likely due to the life history characteristics of peach blossom jellyfish and habitat fragmentation.

The *C. sowerbyi*, a cnidarian that has lived on Earth for at least 550 million years (He and Xu, 2003), shows alternating generations of sexual and asexual reproduction (Chen *et al.*, 2010), specifically in the order jellyfish, floating larvae, polyp, and jellyfish again. After the sperm and eggs produced by sexually mature jellyfish combine, they develop into 0.14-0.31 mm rod-shaped larvae that float on waves (Chen *et al.*, 2010), and they float in the water for 3-5 days before attaching to the bottoms of rivers, lakes and ponds to develop into water pods. The hydra body produces more jellyfish bodies in the form of budding or transverse splitting. Notably, when the environment is not suitable, the polyp shrinks and secretes a hard protective film to encase the body and form a dormant structure (podocyst) that can resist the unsuitable external environment (Peter and Slatkin, 2015); these dormant bodies can allow the individuals to survive until conditions are again suitable. Upon disruption by plants, animals, trampling (such as by birds) or human factors and when the environmental conditions are suitable, the dormant body develops into a polyp, and the life cycle can continue (Wang *et al.*, 2010).

Table VI. Analysis of variance of molecular variance (AMOVA) based on the *COI* gene.

Source of variation	Gene	df	Sum of squares	Variance component	Percentage of variation/%
Among populations	*COI*	2	1.143	0.01514 Va	5.27
Within populations	*COI*	61	16.067	0.27232 Vb	94.73
Total	*COI*	63	17.210	0.28746	
F_{st}	*COI*	0.05268			

Based on the characteristics of the life history of peach blossom jellyfish, we believe that there are three main reasons for the low overall nucleotide diversity of this freshwater jellyfish at the three sampling points: (1) At these three sampling points, dormant peach blossom jellyfish may have always been present or may have arrived from elsewhere. AMOVA also showed that 94.73% of the genetic variation occurred within the populations, so these podocyst were most likely derived from an ancestral group. This leads to a large amount of peach blossom jellyfish inbreeding, resulting in inbreeding depression. Inbred decline has resulted in low nucleotide diversity in the three jellyfish populations. (2) The peach blossom jellyfish is extremely demanding on the environment (Ou *et al*., 2013), so it is extremely rare for peach blossom jellyfish to appear continuously at a sampling point, and in some places, they occur only once every few decades (Liu and Chen, 2004). The genetic variability of organisms accumulates in response to environmental changes (Nevo, 2001), and although the peach jellyfish is an ancient organism, it will remain dormant for a long period of time after a small change in the environment, Low rate of gene mutations preventing the accumulation of nucleotide diversity. (3) Peach blossom jellyfish usually appear in unisexual populations, so sexual reproduction is extremely rare (Pennak, 1989), and long-term, large-scale asexual reproduction leads to a lack of heterozygosity in offspring, which in turn leads to low genetic diversity.

In addition, habitat fragmentation is one of the most important factors affecting the genetic diversity of animals and plants (Sinclair and Hobbs, 2009). Although Guizhou province has a good ecological environment compared to those of other developed provinces in China, due to the extremely specific habitat requirements of the peach jellyfish, even a pot of laundry water can cause the jellyfish to enter dormancy (Ou *et al*., 2013). Human activities in Guizhou province still have a nonnegligible impact on peach jellyfish habitat, which makes the peach jellyfish population increasingly smaller and in turn intensifies the founder effect. The founder effect refers to the phenomenon in which a few individuals from a large population or a single fertilized female move into a new environment to establish a new population. The founders often carry only a small number of gene combinations from the gene pool of the parent population; however, the gradual loss of genetic diversity in organisms is caused by a series of founder events as populations move into previously uninhabited areas (Peter and Slatkin, 2015). Therefore, the dormant jellyfish continued to enter new environments, exacerbating the founder effect, which is one of the reasons for the overall low nucleotide diversity observed in the three populations. On the other hand, genetic drift is inversely proportional to population size, and the population of *C. sowerbyi* is shrinking, which aggravates genetic drift, increases the likelihood of alleles being lost, and leads to low genetic diversity.

Genetic differentiation between populations of peach blossom jellyfish in Guizhou province

The genetic distance between populations is the main indicator of the degree of population differentiation (Xiang *et al*., 2013). Shaklee *et al*. (1982) proposed that the genetic distances at the genus, species and population levels were 0.9, 0.3, and 0.05, respectively. The genetic distances between the three *C. sowerbyi* populations in this study were all 0.001, on the basis of which the three populations could be identified as one population.

Balloux and Nicolas's (2002) criteria for population genetic differentiation are as follows: No genetic differentiation between populations when $F_{st}<0.05$, and a lower degree of genetic differentiation between populations when $0.15> F_{st} >0.05$; when $0.25>F_{st}>0.15$, the degree of genetic differentiation between populations is moderate. The WS and HD populations had no genetic differentiation, while XW showed low and moderate genetic differentiation from HD and WS, respectively.

Geographic isolation is an important factor leading to genetic differentiation among populations, and the degree of genetic differentiation increases with geographic distance (Wright, 1943). The WS and HD populations are in the same city, Tongren, and the two sampling points are geographically close, resulting in a lack of genetic differentiation. The XW population is geographically far from the other two populations and thus has a high degree of geographical isolation, resulting in different degrees of genetic differentiation. The NJ tree (Fig. 1) also supports geographic distance models, such as the closest groups of relatives at the two sampling points of Wanshan Town and Zodiac Township. Notably, the XW population is geographically distant from the WS and HD populations, and the motility of the peach blossom jellyfish is not strong, but the gene flow $N_m>1$, indicating that some genetic exchange occurs. This is likely caused by the combination of the aquatic environment and the life history of peach blossom jellyfish in Guizhou province. Guizhou province is situated on a typical karst landform, a quarter of China's groundwater is distributed in the karst region (Lang, 2005), and Guizhou province has abundant groundwater resources, such as caves and underground dark rivers. Some aquatic animals in Guizhou province, such as giant salamanders (Wu Mata School), can come into contact through underground rivers and achieve long-distance gene exchange. The dormant bodies produced by jellyfish are transmitted through underground rivers and karst water through aquatic plants, animals, bird feet or human factors, which provide conditions for gene exchange among the XW, WS and HD populations.

The genetic differentiation of the three Soxhlet jellyfish populations was very low, and the genetic distances of the three populations were between 0.0000 and 0.0010. Interestingly, the HD and WS populations had high haplotype diversity and low nucleotide diversity. Haplotype diversity accumulates faster than nucleotide diversity in a short period of time (Bowen *et al*., 2001), and biological populations can show high haplotype diversity and low nucleotide diversity because of the recent expansion of a small number of effective populations (Xiang *et al*., 2013). This phenomenon of high haplotype diversity and low nucleotide diversity in biological populations has been reported in marine organisms in the Pacific Ocean (Li *et al*., 2009; Zhao *et al*., 2015). We found large WS and HD populations during sampling, especially the Huangdaoxiang population (Supplementary Fig. 2). Different from the conditions of the XW group, there was an inflow of mountain spring water at these two sampling points; fish are raised in the water, the water body is relatively turbid, the degree of nutrition is high, and the food of peach blossom jellyfish is abundant. The peach blossom jellyfish expanded rapidly from a small population, so the genetic diversity indexes showed high haplotype diversity and low nucleotide diversity.

The method of survival of lower invertebrates

Combining this study with the life history characteristics of jellyfish, we can see that, as an ancient, basal organism, although their ability to resist environmental changes is weak, jellyfish can enter long-term dormancy to cope with unsuitable environments. In this state, the jellyfish may wait or undergo dispersal until an environment suitable for survival is encountered, and the life cycle continues. Many lower invertebrates may be much better able to survival than higher animals due to their dormant eggs or dormant periods, as well as a variety of transmission methods, and it is much more difficult to determine their endangerment (Song *et al*., 2002). For example, the fairy shrimp (Crustacea, a general term for all members of the order Anostraca), which is an ancient and basal species like the peach blossom jellyfish, can produce eggs that are resistant to freezing and drought and can survive in water. They may survive for years or even decades (Li *et al*., 2018). Interestingly, once they encounter a suitable environment, they will initiate rapid development and reproduce in large quantities to provide progeny with more superior basic conditions. The dormant eggs or dormant bodies can continue to wait for a suitable environment and eventually reach their ultimate goal-reproduction.

Conservation suggestions for C. sowerbyi

The peach blossom jellyfish is the only jellyfish in the world that can survive in fresh water, and we have a responsibility to protect its population, with the following recommendations:

1. Establish peach blossom jellyfish ecological protection points in Guizhou Province.
2. Carry out research on artificial breeding of peach blossom jellyfish and expand artificial populations. After the initial success of artificial breeding, research on ex situ conservation is actively carried out to appropriately expand the distribution area to facilitate the reproduction of its race.
3. Carry out ecological research on peach blossom jellyfish. The reasons for the emergence and survival of peach blossom jellyfish are analyzed, which provides a strong scientific basis for better protecting peach blossom jellyfish and protecting the ecological environment.

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IRB approval and ethical statement

The animal study protocol was approved by the Experimental Animal Ethics Committee of Guizhou University (protocol code EAE-GZU-2022-E028 and date of approval 10 August 2022).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20221016141056

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Exploration of the Genetic Diversity and Differentiation of *Craspedacusta sowerbyi* in Guizhou Province

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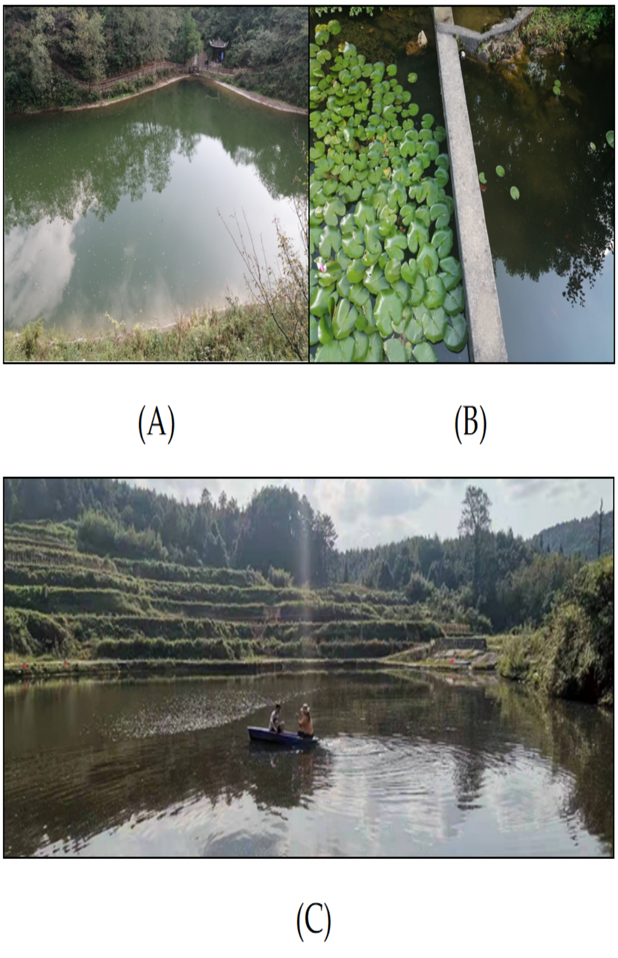

Supplementary Fig. 1. Sample water figure (A, B, and C represent the XW, WS, and HD sampling points, respectively.

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Supplementary Fig. 2. Size of the HD population.